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CERTIFICATE

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I hereby certify that annexed is a true copy of the Provisional Specification as filed on 3 December 2003 with an application for Letters Patent number 529936 made by COLIN RICHARD GREEN and WILDA TETUANUI LAUX-FENTON.

Dated 10 February 2005.

Neville Harris
Commissioner of Patents, Trade Marks and Designs

COMMISSIONER OF PATENTS, TRADEMARKS & DESIGNS
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529936

NEW ZEALAND
PATENTS ACT, 1953

PROVISIONAL SPECIFICATION

TISSUE ENGINEERING OR REMODELLING

We, **COLIN RICHARD GREEN**, a New Zealand citizen of 3/4 Crescent Road, Epsom, Auckland, New Zealand and **WILDA TETUANUI LAUX-FENTON**, a New Zealand citizen of 23 Jordan Road, Mangere, Auckland, New Zealand, do hereby declare this invention to be described in the following statement:

The present invention relates to the field of tissue engineering (bioengineering), or remodelling particularly, although by no means exclusive to the field of corneal engineering or remodelling by manipulation of cell communication.

BACKGROUND

Tissue or organ failure due to illness or injury is a major health problem worldwide with little option for full recovery other than organ or tissue transplantation. However, problems finding a suitable donor mean that this option is not available to the majority of patients.

One alternative treatment which is emerging is tissue engineering or remodelling whereby synthetic or semisynthetic tissue or organ mimics that are either fully functional or which grown in the required functionality are being used as replacements.

One area in particular that this technology is becoming increasingly important for is in the cornea of the eye. The cornea is the transparent tissue which comprises the central one sixth of the outer tunic of the eye. Its unified structure and function provide the eye with a clear refractive interface, tensile strength, and protection from external factors. The cornea is built from three different main layers of cells: the epithelium, the stroma and the endothelium (Pepose and Ubels, 1992; Spencer, 1996). In addition, the Descemet's membrane, the Bowman's layer, and the basement membrane are structures that are derived in some ways from one of these main cellular layers.

The corneal epithelium is the layer in direct contact with the outside world. It is a stratified squamous, non keratinised structure with a thickness ranging from 40 to 100μm, in rats and in humans, respectively. It is comprised of a superficial zone, usually formed by two to three layers of flat squamous cells; a middle zone, formed by two or three layers of polyhedral wing cells; and a basal zone consisting of a single row of columnar cells. The stratified corneal epithelium is characterised as a 'tight' ion transporting functional syncitium which serves both as a protective barrier to the ocular surface, as well as an adjunct fluid secreting layer assisting the corneal endothelium in the regulation of stromal hydration, and thereby contributing to the maintenance of corneal transparency. The unique and specialised qualities offered by the corneal epithelium have been proven to be essential for the operation of the cornea as the principal refractive element of the eye. It is therefore important that its stratified structure be maintained irrespective of any environmental stresses.

Trauma to the surface of the cornea frequently happens, for example minor scrapes, eye infections and diseases, chemical or mechanical accidents and surgical practice can all damage the cornea.

Corneal regeneration after trauma is complex and not well understood. It involves the regeneration of three tissues: the epithelium, the stroma and the endothelium. Three main intercellular signalling pathways are thought to coordinate tissue regeneration: one mediated by growth factors (Baldwin and Marshall, 2002), cytokines (Ahmadi and Jakobiec, 2002) and chemokines (Kurpakus-Wheater et al., 1999); one mediated by cell-matrix interactions (Tanaka et al., 1999); and one mediated by the connexin family of channel forming proteins.

WO 00/44409 describes the use of antisense (AS) oligodeoxynucleotides to downregulate connexin expression to treat local neuronal damage in the brain, spinal cord or optic nerve, in the promotion of wound healing and reducing scar formation of skin tissue following, for example, cosmetic surgery or burns.

Connexins form part of gap junctions involved in cell-cell communication. To date, 20 human and 19 murine isoforms have been identified (Willecke et al., 2002) and this diversity suggests that each different connexin protein may be functionally specialised. In addition, different tissues and cell types show characteristic patterns of connexin protein expression, although there is some overlap.

Corneal tissue has been shown to alter its connexin protein expression pattern following injury. However, contrary to the skin, very little is known about these changes.

The corneal regeneration process post-trauma can result in the loss of corneal clarity and therefore influence the outcome of refractive surgery. Present treatments for damaged cornea generally include corneal transplant or use of corneal cells/tissue for reconstruction.

However, the prevalence of refractive surgery for correction of myopia such as photorefractive keratectomy (PRK) and laser in situ keratomileusis (LASIK) has led to shortage of suitable cornea for transplant for tissue reconstruction after surgery or disease processes and for tissue manipulation in vivo to engineer changes. In addition, approximately 5% of patients undergoing laser surgery experience unexpected outcomes.

It is an object of the present invention to go some way toward overcoming these problems and/or to provide the public with a useful choice.

SUMMARY OF THE INVENTION

According to a first aspect the present invention provides a method of tissue engineering or remodelling comprising administering an agent that modulates direct cell-cell communication to the site on a tissue or organ to be remodelled or during ex vivo growth of a tissue for transplant.

Preferably, the agent is selected from the group comprising an antisense polynucleotide molecule, a deoxyribozyme, a morpholino oligonucleotide or RNAi or a related molecule which is capable of reducing translation of a connexin protein; or a mimetic peptide or non-specific uncoupler (such as octenol, glycerrhetinic acids, heptanol etc).

Preferably the agent is selected from an antisense polynucleotide molecule, a deoxyribozyme, a morpholino oligonucleotide or RNAi which is targeted to connexin 43, 26, 37, 30 or 31.1.

Preferably the tissue to be remodelled is a cornea or related tissue such as skin which expresses a similar range of connexin proteins (Laux-Fenton et al, 2003).

According to the second aspect, the present invention provides a method of preventing or treating corneal defects comprising administering to the cornea an agent selected from the group comprising an antisense polynucleotide molecule, a deoxyribozyme, a morpholino oligonucleotide, RNAi or a related molecule which is capable of reducing translation of a connexin protein; or a mimetic peptide or non-specific uncoupler (such as octenol, glycerrhetinic acids, heptanol etc).

The corneal defect may be any naturally occurring defect (such as myopia) or a defect caused by illness, injury or laser surgery.

In a third aspect, the present invention provides a use of an agent selected from an antisense polynucleotide molecule, a deoxyribozyme, a morpholino oligonucleotide an RNAi or a related molecule, which is capable of reducing translation of a connexin protein; or a mimetic

peptide or non-specific uncoupler (such as octenol, glycerrhetinic acids, heptanol etc) in the manufacture of a medicament for tissue re-modelling or tissue engineering.

Preferably, the agent is an antisense polynucleotide against connexion 43, 26, 37, 30 or 31.1 and the tissue to be re-modelled is a cornea.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows in vivo confocal microscopic images of corneas 12 hours post-photorefractive keratectomy in control and AS ODN treated eyes;

Figure 2 shows histological examination of corneal remodelling in control and AS ODN treated rat corneas 24 hours after excimer laser photoablation;

Figure 3 shows expression of connexin43 protein in control and AS ODN treated corneas at 24 hours after excimer laser ablation;

Figure 4 shows myofibroblast labelling 1 week after surgery using alpha smooth muscle actin antibodies;

Figure 5 shows laminin-1 labelling of control and connexin43 AS ODN treated corneas 24 hours (A – D) and 48 hours (E – H) after photorefractive keratectomy;

Figure 6 shows a schematic diagram of laminin-1 irregularity quantification; and

Figure 7 shows immunohistochemical labelling for connexins 26 and 43 in control cultures (A) and following three treatments with anti-connexin43 ODNs over a 24 hour period (B), and after connexin31.1 specific antisense treatment (C,D).

DETAILED DESCRIPTION OF THE INVENTION

As broadly defined above, the focus of the invention is the use of an agent for site-specific down-regulation of connexin expression. This will have the effect of reducing direct cell-cell

communication at the site at which connexin expression is downregulated, which gives rise to tissue engineering or tissue remodelling applications as described below.

Regulation of direct cell-cell communication is based on active molecules that directly or indirectly reduce coupling between cells in tissues. Such molecules include polynucleotides such as antisense deoxynucleotides, morpholino nucleotides, RNAi and deoxribozymes targeted to specific connexio isoforms which result in reduced translation of the protein isoform and interfere with the function of cell gap junctions. Alternatively, mimetic peptides or non-specific uncouplers (such as octanol, glycerhetinic acids, heptanol etc) may be used.

Connexin targets will vary depending upon the type of tissue to be engineered or remodelled, as will be appreciated by a skilled worker.

The present invention finds particular application in tissue engineering and remodelling of the cornea, particularly to aid in correcting visual defects in conjunction with laser surgery, for in vitro corneal engineering and for direct eye treatments where remodelling of the cornea is desired.

For such corneal applications, the connexins targeted by the active molecules described herein are connexin 43, 26, 37, 30 and 31.1 which have all recently been shown to be expressed in the cornea (Laux-Fenton et al., 2003).

Particularly preferred is the use of antisense oligodeoxynucleotides (AS ODNs) targeted towards specific connexin proteins, in a method of tissue engineering or remodelling.

For corneal engineering or remodelling, it is contemplated that ODNs targeted towards connexin 43, 26, 37, 30 and /or 31.1 will be particularly suitable for use in the present invention, either alone or in combination.

The ODNs for use in the invention will generally be unmodified phosphodiester oligomers. They will vary in length but with a 30 mer ODN being particularly suitable.

The precise sequence of the ODN used in the invention will depend upon the target connexin protein. For connexin43, the applicants have found ODNs having the following sequences to be particularly suitable:

5 ' GTA ATT GCG GCA AGA AGA ATT GTT TCT GTC 3';

5' GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC 3'; and

5' GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT 3'

ODNs directed to other connexin proteins can be selected in terms of their nucleotide sequence by any convenient and conventional approach. For example, the computer programmes MacVector and OligoTech (from Oligos etc. Eugene, Oregon, USA) can be used. For example, ODNs for connexins 26, 37, 30 and 31.1 have the following sequences:

5' TCC TGA GCA ATA CCT AAC GAA CAA ATA 3' (connexin26)

5' CAT CTC CTT GGT GCT CAA CC 3' (connexin37)

5' CTG AAG TCG ACT TGG CTT GG 3' (connexin37)

5' CTC AGA TAG TGG CCA GAA TGC 3' (connexin30)

5' TTG TCC AGG TGA CTC CAA GG 3' (connexin30)

5' CGT CCG AGC CCA GAA AGA TGA GGT C 3'(connexin31.1)

5' AGA GGC GCA CGT GAG ACA C 3' (connexin31.1)

5' TGA AGA CAA TGA AGA TGT T 3' (connexin31.1)

Once selected, the ODNs can be synthesised using a DNA synthesiser.

For use in the invention, the ODNs require site-specific delivery. They may also require delivery over an extended period of time. While clearly the delivery period will be dependent upon both the site at which the tissue engineering or remodelling is to be induced and the tissue type, continuous delivery for 24 hours or longer may often be required, and may be

achieved by inclusion of the ODNs in a sustained release formulation as described in WO 00/44409.

By regulating direct cell-cell communication in corneal tissue using ODNs to connexin43, it has been found to be possible to engineer stromal re-modelling, engineer basal lamina deposition between new epithelium and underlying stroma, and to engineer epithelial remodelling.

The invention will now be described with reference to the following experimental section which will be understood to be provided by way of illustration only and not to constitute a limitation on the scope of the invention.

EXPERIMENTAL

EXPERIMENT 1 – IN VIVO ANALYSIS

Materials and Methods

Laser treatment

Female Wistar rats (d32-34) were raised under conditions consistent with the ARVO Resolution on the Use of Animals in Research. Animals were anaesthetised by administrating a 1:1 mixture of Hypnorm™ (10mg/ml, Jansen Pharmaceutica, Belgium) and Hypnovel® (5mg/ml, Roche products Ltd, New Zealand) at a dose of 0.083ml/100g body weight in the peritoneum of the animal.

Excimer laser treatment was performed through the intact epithelium using a Technolas 217 Z excimer laser (Bausch & Lomb Surgical, USA). The eye was centered at the middle of the pupil and ablation was performed with the following parameters: treatment area was of 2.5mm diameter and of 70μm depth. This resulted in the removal of a small thickness of the anterior stroma and of the whole epithelium. All surgical procedures were performed by Professor Charles McGhee, assisted by Dr Christina Grupcheva and Mrs Jennifer McGhee from the Department of Ophthalmology, Faculty of Medical and health Sciences, University of Auckland. Excimer laser treatment was preferentially used to produce reproducible lesions

and investigate the effects of connexin43 AS ODNs on corneal remodelling and engineering after trauma.

Following surgery, all animals were placed in individual cages and closely monitored for any discomfort. Post-surgical in vivo evaluation was achieved using a slit lamp biomicroscope and/or a slit scanning in vivo confocal microscope.

Slit scanning in vivo confocal microscopy

Prior to, and following corneal laser treatment, each animal was observed clinically using a Confoscan 2 (Fortune Technologies America, USA) slit scanning in vivo confocal microscope. The Confoscan 2 is a variant of slit scanning technology with the distinct advantage of direct digitisation of the images at the time of acquisition. Animals were anaesthetised and each of them was placed onto a specially designed platform that was adjusted at the level of the in vivo confocal microscope objective lens in front of the acquisition head.

The slit scanning in vivo confocal microscope allows optical dissection of the living cornea at different levels through the whole corneal thickness. The examination starts from the endothelium and the number of the antero-posterior sections depends upon the customised settings. The slit scanning technology utilises an objective lens that moves back and forward along the axis perpendicular to the examined area. In brief, the hardware consists of a halogen lamp (100W/12V), two slits, two tube lenses, a front objective lens, and a highly sensitive digital (CCD) camera. Prior to scanning, a drop of Viscotears (CIBAVision Ophthalmics) is placed on the tip of the objective lens as an immersion substance. During scanning, the eye of the animal is held wide open and orientated so that the corneal plane is always perpendicular to the optical axis of the magnification lens (40x, N.A 0.75). The image acquisition time is approximately 14 seconds. The gel, not the objective lens contacts the eye at all times. For the rat cornea, up to 250 sequential digital images were obtained per examination, and were directly saved to a hard disk drive. Acquisition parameters were adjusted during the preliminary experiments and were kept constant for all subsequent experiments. They were as follows: the light intensity was decreased to half the intensity generally used for human patients, four passes (one pass is considered as being a full back and forward movement) were

used, and a 400μm working distance was selected. For the rat cornea, centration is facilitated by clear visualisation of the pupil, which provides very good topographical repeatability.

Data Analysis

In vivo confocal images

All images acquired with the slit scanning in vivo confocal microscope were stored onto the hard disc drive and subsequently analysed by NAVIS proprietary software (Confoscan 2, Nidek Co Ltd).

Stromal dynamics were evaluated following stereological principles. Cell counts were recorded at the anterior and posterior stromal positions. The main stereological component was provided by the in vivo confocal microscope itself as it functions as an optical dissector (a probe that samples with equal probability particles in space). Indeed, the in vivo confocal microscope provides thin optical slices of specified volume, with each being a dissected tissue sample. As a result, counting stromal cells consists in choosing a pair of frames (consecutive pictures recorded by the in vivo confocal microscope), one frame having particles (stromal cells) in focus, and the co-frame showing a defocused but recognisable image of the same particles (optical shadows). The number of cells (n) is recorded from the clearest frame in a defined area A (μm^2). The distance d (μm) between the two frames is also recorded. The number of cells per unit volume (V) therefore equals to:

$$V = \text{Number of cells (n)} / d\ (\mu m) \times A\ (\mu m^2)$$

Ex vivo confocal images

Appropriate corneal sections were immunohistochemically stained with different markers for different purposes. Staining with the nuclear stain Hoechst 33 258 was used to estimate the number of epithelial and stromal cells at the central or the peripheral cornea. For this purpose using AnalySIS® 3.2 software (Soft Imaging System, USA), the area of interest was first freehand drawn onto the TIFF file image of the appropriate region of the cornea and the value of the area was automatically given by the software. Using the manual count option, cells were then counted within that area and expressed per unit area.

Antisense application

30% Pluronic acid gel (BASF Corp) in phosphate buffered saline (molecular grade water) was used to deliver unmodified a1 connexin (connexin43) specific antisense ODNs to the subconjunctiva of anaesthetised rats following photorefractive keratectomy. In a pre-trial using an FITC tag, this formulation was shown to remain in the anterior chamber of the eye for up to 24 hours (not shown).

The antisense molecule used in these experiments was DB1 (GTA ATT GCG GCA GGA ATT GTT TCT GTC). Addition of an FITC tag to DB1 ODN, viewed using confocal laser scanning microscopy, demonstrated intracellular penetration of the probe.

The ODN was applied at a 2μM final concentration.

Monitoring tissue engineering or remodelling effects

After antisense application, the corneas were examined using a slit scanning in vivo confocal microscope at 2h, 12h, 24h, 48h, 72hr, 1 week and 2 week post laser surgery. Control rats received laser surgery only.

Table 1 summarises the number of corneas investigated at each time point.

Table 1. Number of control (C) and AS ODN treated corneas used for the in vivo follow-up using slit scanning in vivo confocal microscopy.

ODN= AS ODN treated eyes (single administration after laser surgery)

	Within 2 hr surgery	12hr post-surgery	24hr post-surgery	48hr post-surgery	72hr post-surgery	1 week post-surgery	2 weeks post-surgery
Number of eyes (n)	18 C 18 ODN	10 C 10 ODN	18 C 18 ODN	10 C 6 ODN	6 C 6 ODN	4 C 4 ODN	5 C 5 ODN

Each cell layer of the cornea was analysed and the cell type, number and appearance compared between the control and ODN treated groups.

Results

Re-epithelialisation:

Treatment with anti-connexin43 ODNs promoted epithelial recovery. In 90% of AS ODN treated corneas, sliding epithelial cells were observed within 12 hours after PRK laser surgery, compared to none in controls (Figure 1B). At this stage only static endothelial cells were

present in 30% control corneas (Figure 1A) By 24 hours epithelial cells were seen in all controls and antisense treated corneas but 72% of treated versus 61% of controls showed actively sliding cells implying that re-epithelialisation is proceeding faster in the connexin43 specific AS ODN treated corneas than in controls.

Stromal cell densities:

Using a paired samples t-test with repeat measures to compare cell densities in the anterior and posterior stroma within each group as a function of time and a Mann Whitney non parametric statistical test to compare stromal cell counts between control and ODN treated corneas at the selected time points, the only statistically significant results were found at 24hr post-laser surgery (Table 2). At this time point, in the control and ODN treated groups stromal cell density in the anterior stroma has increased considerably compared to the pre-surgery values (p value < 0.05). In the posterior stroma of control corneas, stromal cell density has also increased compared to the pre-surgery value (p value < 0.05) whilst in the posterior stroma of ODN treated corneas, stromal cell density is not statistically significantly different from the pre-surgery value (p value > 0.05). When comparing stromal cell density between the two groups at the anterior and posterior stroma, the ODN treated corneas always showed lower stromal cell densities than the control corneas (p-value < 0.05). This supports the hypothesis that a smaller number of cells are involved in stromal re-modelling or engineering in the ODN treated corneas compared to the control corneas. This is the first quantitative report showing that application of anti-connexin43 ODNs reduces hypercellularity at the site of surgery. Ex vivo histochemical analysis (Experiment 2) shows that this hypercellurity is associated with myofibroblasts which induce unwanted stromal matrix remodelling and scarring (Ahmadi and Jakobiec, 2002).

Table 2. Stromal cell counts in control and AS ODN treated corneas prior to and 24hr following photorefractive keratectomy. Cell densities are given as means followed by standard deviations.

Treatment	Time points	Anterior stromal cell count (#cells/mm^3)	Posterior stromal cell count (#cells/mm^3)
Control	Pre-surgery	36469 ± 11122 (n=17)	33909 ± 8753 (n=17)
ODN	Pre-surgery	36769 ± 10932 (n=17)	34382 ± 8667 (n=14)
Control	24hr post-surgery	144643 ± 60989 (n=17)	46901 ± 26964 (n=17)
ODN	24hr post-surgery	93468 ± 53548 (n=17)	33510 ± 11350 (n=14)

EXPERIMENT 2 – EX VIVO ANALYSIS

Materials and Methods

Histology

Tissue collection and fixation

Appropriate numbers of animals (Wistar rats) were terminated at selected time points following photorefractive keratectomy and DB1 anti-connexin43 ODNs were administed to anaesthetised rats as described in experiment 1 above and corneal sections were prepared for histological analysis. Control rats had received laser surgery only. Whole eyes and control tissues were rinsed in Oxoid PBS prior to embedding in Tissue-Tek® OCT (Sakura Finetek, USA) and freezing in liquid nitrogen. When necessary (for the use of some antibodies), frozen tissues were later fixed in cold (-20°C) acetone for 5min after being cryocut..

Tissue cutting

The procedure for cryosectionning was as follows: frozen blocks of unfixed tissue were removed from -80°C storage and placed in the Leica CM 3050S compact cryostat for about 20min to equilibrate to the same temperature as the cryostat (ie. -20°C). When equilibration of the tissue was achieved, the specimen was mounted onto a specimen disc with Tissue Tek® OCT. Sections of 12µm (for H/E staining) or 25µm thick (for immunolabelling) were cut and placed on Superfrost®Plus slides (Menzel-Gleser, Germany). Immediately following cryocutting, tissue blocks were placed back to -80°C storage and slides supporting cryosections were either used immediately or stored at -80°C. Sectioning occurred parallel to the optical axis of the eye.

Haematoxylin/Eosin (H/E) staining and nuclear Staining

Slides were placed in glass racks to facilitate immersion in a series of different staining reagents. It was also important to agitate racks when placing them into reagents to break surface tension and to drain them between each solution change. Prior to Gill's II Haematoxylin/Eosin staining, slides that were stored at -80°C were first warmed up to room temperature for 1-2min, then either fixed in cold acetone first and/or immediately hydrated with a quick dip in tap water. Slides were stained in Gill's II Haematoxylin for 2min, after which excess stain was rinsed off in tap water. Stain differentiation was achieved by dipping in Scott's tap water substitute (STWS) for 4sec. A rinse in running tap water for 1min was then performed before staining in 1% eosin for 30 consecutive dips. Finally, sections were quickly rinsed in tap water, dehydrated through 95%, 100% EtOH, cleared in xylene, and mounted with DPX mounting medium (Sigma). For nuclear counter staining (in parallel with H/E or immunohistochemical analysis) Hoechst 33 258 (Sigma) was used. Measurement of cornea thickness was carried out on H/E stained sections.

Immunohistochemistry

Sections were immunolabelled for connexin43 using a site specific monoclonal antibody, for myofibroblasts using an antibody recognising alpha smooth muscle actin, for basal lamina deposition with an anti-laminin-1 antibody. In addition anti-vimentin antibodies were used to differentiate stromal keratocytes from myofibroblasts and a Ki-67 antibody was used to show cell proliferation.

Results

Ex vivo histological analysis

Results showed that lesions made by excimer photoablation had closed by 24hr post-surgery (Figure 2). The typical invasion of the stroma by mononucleated/multinucleated and/or round, ovoid cells at the periphery and at the centre of the cornea was observed in both groups, most pronounced at 24hr post-surgery, but with the antisense ODN treated group showing a significantly smaller number of these cells than the control group (Figure 2A,B,C). This parallels the findings from the in vivo confocal photomicrographs in experiment 1. In the control corneas the epithelium thickness was variable as seen in Figure 2 at the site of laser induced lesion (A, B), and in the stoma beneath the ablated area (A,B) and in the peripheral stroma (C) there was an extensive invasion by round cells (hypercellularity). Also observed was a pronounced stromal oedema in B and C. In the antisense ODN treated

corneas the epithelium was of even thickness (D,E) and in the central region (D) and in the peripheral stroma (E) there was little sign of stromal oedema. Moreover, in the stroma there were few round cells present. Scales bars in Figure 2 represent 20 microns.

Changes in stromal thickness following treatment with connexin43 ODNs after laser treatment are shown in Table 3 comparing changes in stromal thickness between control and ODN treated corneas. Stromal thicknesses were measured from appropriate histological stained sections. Statistical analysis of the data obtained for the ODN treated group using a paired samples t-test showed that at all three time points investigated (24hr, 48hr and 72hr post-surgery) central stromal thickness is statistically significantly thinner than pre-surgery value (p values <0.05) and peripheral stromal thickness is not significantly different from pre-surgery values. In contrast, control corneas show significant stromal swelling (oedema) (Figure 2) in both central and peripheral cornea (where the stroma doubles in thickness compared to pre-surgery values).

Table 3. Changes in stromal thickness following excimer laser surgery in control and AS treated corneas.

Normal, i.e. cornea which is not subjected to surgery, had a central stromal thickness of 250μm but excimer laser surgery was used to remove 70μm of corneal tissue (including the epithelium and part of the stroma). The normal corneal epithelium is 50μm thick (on average) and therefore 20μm of stromal tissue was removed by laser surgery. Therefore, to statistically compare the central stromal thickness at 24hr, 48hr and 72hr post-wounding to the pre-surgery central stromal thickness, an adjusted thickness loss and a central pre-surgery stromal thickness of 250-20 = 230μm was used.

Treatment	Time points	mean central stromal thickness (μm)	mean peripheral stromal thickness (μm)
Normal (no surgery)	Pre-surgery	250 (n=6)*	110 (n=10)
Control ODN treated	24hr post-surgery	318 (n=6) 190 (n=5)	290 (n=6) 132 (n=5)
Control ODN treated	48hr post-surgery	307 (n=6) 158 (n=5)	206 (n=5) 105 (n=5)
Control ODN treated	72hr post-surgery	292 (n=6) 142 (n=5)	201 (n=6) 99 (n=5)

Reduction in connexin43 expression parallels reduced stromal invasion and reduced epithelial hyperplasia

Microscopial observations showed a reduced level of connexin43 present in ODN treated corneas compared to control corneas. Figure 3 shows combined micrograph images. Top row shows control corneas, the bottom row shows antisense ODN treated corneas. The typical invasion of the stroma by round cells was seen in both groups within 24 hours at the limbal, peripheral and central areas, but with a smaller density in treated corneas. At the limbus in both groups anti-connexin43 was evenly distributed throughout the stroma (A,D) but the treated groups had less label in the periphery (E) compared to controls (B). By this stage connexin43 levels had returned to normal in the epithelium of both groups but control groups showed a scar like stroma (C) or hyperplasia (see figure 4 below) whereas in antisense treated corneas a normal epithelium with normal levels of connexin43 was seen (F). Scale bars A, D, E, F represent 10 microns; B and C represent 20 microns. In these figures connexin43 appears as white punctate labelling with cell nuclei appearing grey. The results shown in Figure 3 suggest that connexin43 protein levels are reduced following treatment with anti-connexin43 ODNs and results in a smaller degree of cell recruitment in the stroma. In addition, only 7% of ODN treated corneas (0% at 24hr post-surgery, 0% at 48hr post-surgery, 20% at 72hr post-surgery) show signs of epithelial hyperplasia compared to 31% control corneas (25% at 24hr post-surgery, 67% at 48hr post-surgery, 0% at 72hr post-surgery). This was assessed on H/E stained and Ki-67 labelled sections.

Myofibroblast labelling

Labelling with vimentin antibodies indicated that the increased cell numbers in the stroma of control corneas compared with AS ODN treated corneas were not of undifferentiated keratocyte origin and labelling was therefore carried out with alpha-smooth muscle actin antibodies. This labelling showed that control corneas had a higher number of myofibroblasts beneath the site of surgery, but also in the surrounding peripheral stroma. This increase in myofibroblast numbers and area affected was evident at 24 hours and persisted over 48 and 72 hours through to at least one week after surgery (Table 4). Figure 4 shows myofibroblast labelling (anti-alpha smooth muscle actin) at 1 week post-laser surgery. A, B, and C are controls; and D, E, and F are antisense treated corneas. By one week post-wounding, in the control corneas, low to moderate numbers of myofibroblasts are present in the anterior half of the peripheral stroma (A), moderate to dense levels are present in the mid-peripheral stromal regions (B), and moderate levels are seen in the anterior half of the stroma in central regions (C). In contrast, in the treated corneas, very low numbers of myofibroblasts are present in

peripheral (D) or mid peripheral (E) stroma and moderate to low numbers in central stroma (F). In some cases in the central stroma, myofibroblasts are concentrated in the area just under the epithelium (not shown). Thus, the increased cell numbers seen in experiment 1 (hyerpcellurarity) and Figure 2 above appears to be due to myofibroblast differentiation and invasion. Myofibroblasts are known to be responsible for scar tissue deposition in the stoma, with reduced crystallin deposition and increased secretion of wound collagen III (Ahmadi and Jakobiec, 2002).

Table 4: Summary of alpha smooth muscle actin labelling for myofibroblast in control and antisense ODN treated corneas.

Time	Locations	Control corneas	AS treated corneas
24hr post-surgery	Periphery	80% D in whole st **20% M in whole st**	**100% M in anterior 1/3 st**
	Mid-periphery	**100% D in whole st**	**100% M in anterior 1/3 st**
	Centre	**100% L in anterior ¾ st**	**80% L in anterior 1/3 st** **20% L below epi**
48hr post-surgery	Periphery	**83% M in whole st** 17% L in whole	**40% M in half anterior st** 60% L in half anterior st
	Mid-periphery	**50% D in whole st** 50% M in whole st	**100% D in half anterior st**
	Centre	**17% L under epi** (hyperplasia) 50% D in whole st **33% M in whole st**	20% absent **80% M in anterior ¾ st**
72hr post-surgery	Periphery	**33% L in anterior half st** 67% M in anterior half st	**40% L in anterior half st** 60% M in anterior half st
	Mid-periphery	**33% L in whole st** 67% D in whole st	**20% L in anterior half st** 80% M in anterior half st
	Centre	17% absent 50% D in whole st **33% M in whole st**	20% absent 40% L in anterior half st **40% M in anterior ¾ st**
1 week post-surgery	Periphery	60% M in half anterior st **40% L in half anterior st**	**100% L in anterior 1/3 st**
	Mid-periphery	**60% D in whole st** 40% M in whole st	**100% L in anterior half st**
	Centre	**60% M in anterior half st** 40% L in anterior half st	**60% M in anterior half st** 20% L in anterior half st 20% M under epi

Numbers of myofibroblasts are quantified as dense (D), moderate (M), low (L) or absent. Percentages refer to proportions of animals affected at the specified levels. st = stroma, epi = epithelium. Significant differences between control and antisense treated corneas are highlighted in bold.

Basal lamina deposition

Following photorefractive keratectomy the basal lamina reforms along with the regrowing epithelium. Labelling with antibodies to laminin-1 shows that the reforming basal lamina is discontinuous and with an irregular epithelial-stromal attachment (Figure 5). At 24 hours controls had little and/or uneven laminin deposition at the edge of the ablated area (A) and

more centrally (B) whereas antisense treated corneas showed a more regular deposition of laminin at both of these regions (C,D). At 48 hours controls still do not have a continuous laminin deposition (E – edge of the ablated area; F – central) and it was very uneven (E). In contrast antisense ODN treated corneas had a continuous and relatively even basal lamina at the wound edge (G) and centrally (H). All scale bars in Figure 5 represent 20 microns. Connexin43 antisense treated corneas formed a denser, more continuous basal lamina within 24 hours with less irregularity.

The laminin irregularity was quantified as shown in Figure 6. The black solid line in Figure 6 represents laminin-1 deposition. For each region the variance was measured as the difference between the top of a hill and the bottom of a valley (A,B,C,D). Control corneas had a mean variance of 6.98 microns compared with 4.74 microns in antisense ODN treated corneas. The difference between the two groups was statistically significant ($p<0.0001$).

EXPERIMENT 3 – EX VIVO TISSUE ENGINEERING

Corneas were placed into an ex vivo organ culture model and specific connexin modulated using antisense ODNs. Two connexins were targeted in these experiments, connexin43 and connexin31.1. Connexin43 downregulation is used to demonstrate that connexins can be regulated in vitro, and connexin31.1 has been targeted as this connexin is expressed in the outer epithelial layers of the cornea in cells about to be shed from the cornea. The aim was to engineer a thickening of epithelial tissue by reducing connexin31.1 expression.

Materials and Methods

30-34 day old Wistar rats were euthanized with Nembutal or carbon dioxide and whole rat eyes dissected. The ocular surface was dissected, disinfected with 0.1mg/ml penicillin - streptomycin for 5 minutes and rinsed in sterile PBS. The whole eye was then transferred onto a sterile holder in a 60mm culture dish with the cornea facing up. The eyes were mounted with the corneal epithelium exposed at the air-medium interface and cultured at 34°C in a humidified 5 % CO_2 incubator in serum free medium (Opti-MEM, Invitrogen) for up to 48 hours. 100 µl of medium was added drop wise to the surface every eight to twelve hours to moisten the epithelium. Medium levels were maintained to the level of limbal conjunctiva.

Antisense oligomers were mixed with 30% (w/w) Pluronic F127 gel (Sigma) on ice to a final 2 μM concentration and 10 μl applied onto the corneas. Each treatment had a sample size of 3 to 4 corneas per experiment. Preliminary experiments showed that double treatments of our positive control, DB1, for 8 hours had little effect on connexin43 protein expression in our corneal culture. Corneas were therefore cultured for 24 hours and connexin specific oligomers applied every 8 hours.

Immunohistochemical labelling was carried out as in Experiment 2 above using antibodies to connexins43, 26 (control) and 31.1. Tissue was also stained with H/E as above. Nuclei were counterstained with 0.2 μM propidium iodide. Images were collected on a Leica TCS 4D or TCS SP2 confocal laser scanning microscope with voltage and offset settings maintained within experimental groups to allow quantification of connexin levels. For quantification four optical slices through 3 microns were processed into a single extended focus optical image using the center of mass topographical projection option on the TCS 4D. Connexin label was quantified using NIH Image (Scion Corp. USA) after thresholding at 90 – 100 pixel intensity on the 256 grey scale image.

Results

In normal corneas that have not undergone surgery, in vitro connexin turnover rates were relatively low compared to tissue remodelling processes in the excimer laser ablated corneas described in experiments 1 and 2 above. Nonetheless, after three treatments with antisense ODNs connexin levels were reduced by over 50% compared with controls (Figure 7 A,B shows connexin43 reduction in AS ODN treated corneas compared with controls). Connexin26 levels remained constant when the connexin43 specific antisense ODNs were applied (indicating that the reduction in connexin levels was specific, not a side effect of the treatment. In these images connexin43 appears as heavier spots in the basal two layers of the epithelium, connexin26 as finer punctate labelling predominantly in layers 2 – 6. Connexin31.1 antisense ODNs reduced levels of connexin31.1 but preliminary results also showed that the epithelial thickness (number of layers) increased within 24 hours (Figure 7 C, D). This increase in thickness was seen using H/E staining (D) and in the immunohistochemically (C) labelled sections.

SUMMARY OF RESULTS

The results described in this work form a basis for the use of connexin specific antisense ODNs to engineer tissues, especially following excimer laser surgery of the cornea, or for in vitro organ culture for tissue engineering and transplantation.

It has specifically been shown that a single treatment with connexin43 specific antisense ODNs following excimer laser photorefractive keratectomy:

(i) promotes epithelial cell movement. At 12hr post-surgery 90% antisense treated corneas but no control corneas show the presence of sliding epithelial cells at the site of a laser induced lesion. Epithelial cells were present in 30% of control corneas but were static / non-sliding. Regulation of direct cell-cell communication can therefore be used to engineer changes in epithelial cell patterning;

(ii) controls hypercellularity associated with myofibroblast differentiation at the site of a laser induced lesion in the 24hr to 48hr post-surgery period. During this period, more control corneas (63%) than antisense ODN treated corneas (39%) show intense hypercellularity in the whole stroma. Regulation of direct cell-cell communication can therefore be used to modulate cell differentiation leading to modification of extracellular matrix;

(iii) controls stromal remodelling reducing haze at the site of a laser induced lesion in the 24hr to 72hr post-surgery period. In this period, more control corneas (64%) than antisense treated corneas (39%) show intense haze in the whole stroma;

(iv) inhibits stromal oedema during the early stages of re-modelling. Regulation of direct cell-cell communication therefore improves outcomes from laser surgery;

(v) reduces cell proliferation in the early stages of re-modelling. Regulation of direct cell-cell communication can therefore be used to regulate cell proliferation during tissue remodelling;

(vi) reduces epithelial hyperplasia by 78% (assessed from 24hr to 72hr post-surgery) enabling engineering of an even epithelium;

(vii) reduces myofibroblast activation up to 1 week post-surgery (and earlier loss of keratocytes). Regulation of direct cell-cell communication enables more precise

control of tissue damage during surgical remodelling, providing improved predictability of outcome and fewer visual defects; and

(viii) results in a more regular and denser epithelial-stromal adhesion matrix during tissue re-modelling. Regulation of direct cell-cell communication can therefore be used to engineer tissue basal laminae.

In addition, the ex vivo corneal culture model used herein indicates that regulation of direct cell-cell communication can be used to engineer tissue in vitro, for example increasing epithelial thickness using connexin31.1 antisense ODNs. This treatment also has implications in vivo, for example in the engineering a thicker cornea for the relief of corneal diseases such as keratoconus (a thinning of the epithelium).

CONCLUSION

The results of this invention show for the first time that active molecules which interfere with cell-cell communication can be used in tissue engineering and remodelling. Specifically, it is shown that antisense deoxynucleotides targeted at connexin proteins can be used in corneal re-modelling especially following corrective laser surgery, as well as for in vivo and in vitro tissue engineering.

This approach has significant potential for improving the outcome of surgical interventions and ameliorating disease processes in the eye, and for tissue engineering.

It will be appreciated that the above description is provided by way of illustration only and that the invention is not limited thereto.

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Figure 1

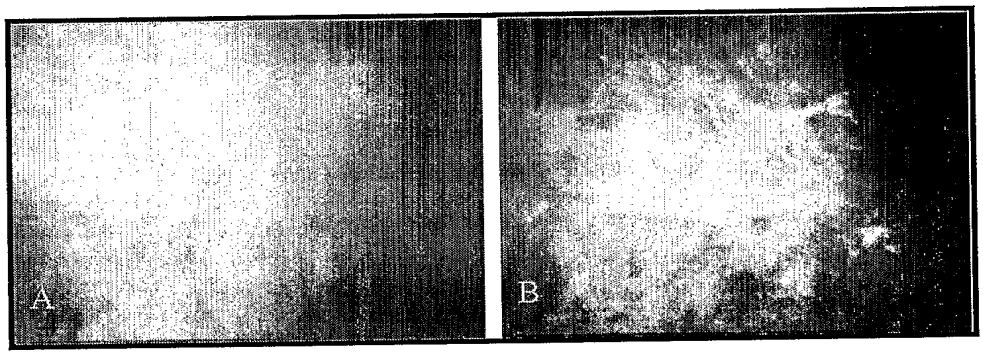

Figure 2

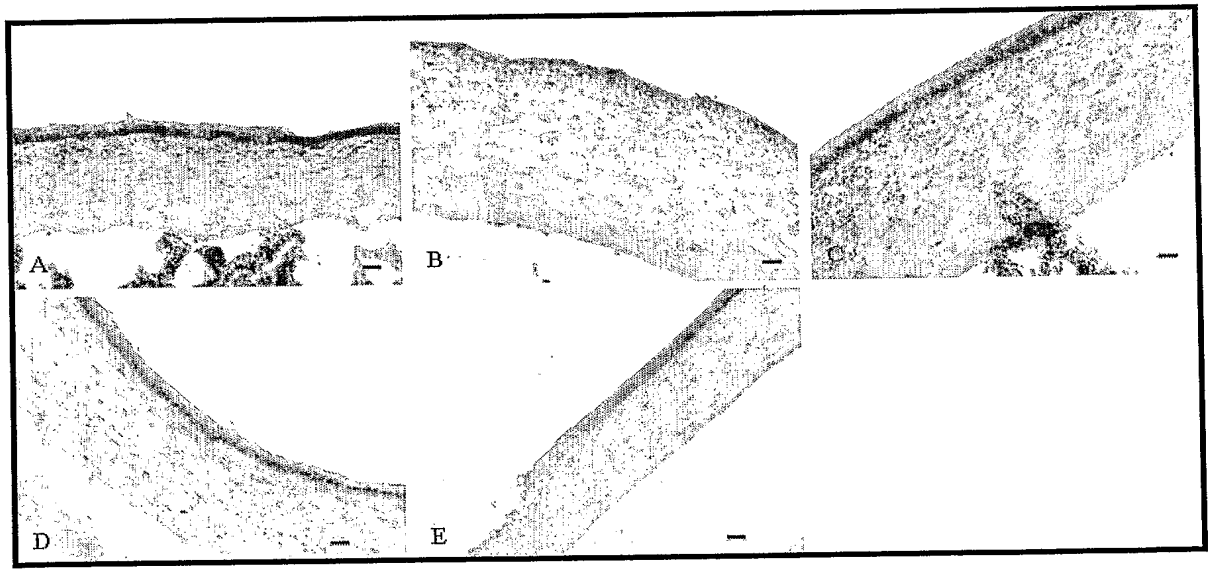

Figure 3

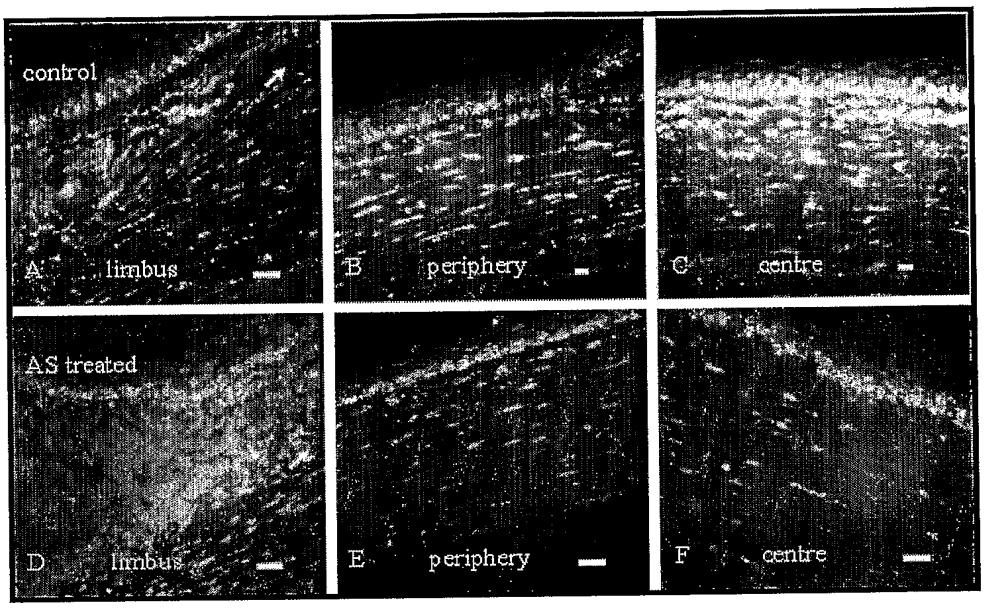

Figure 4

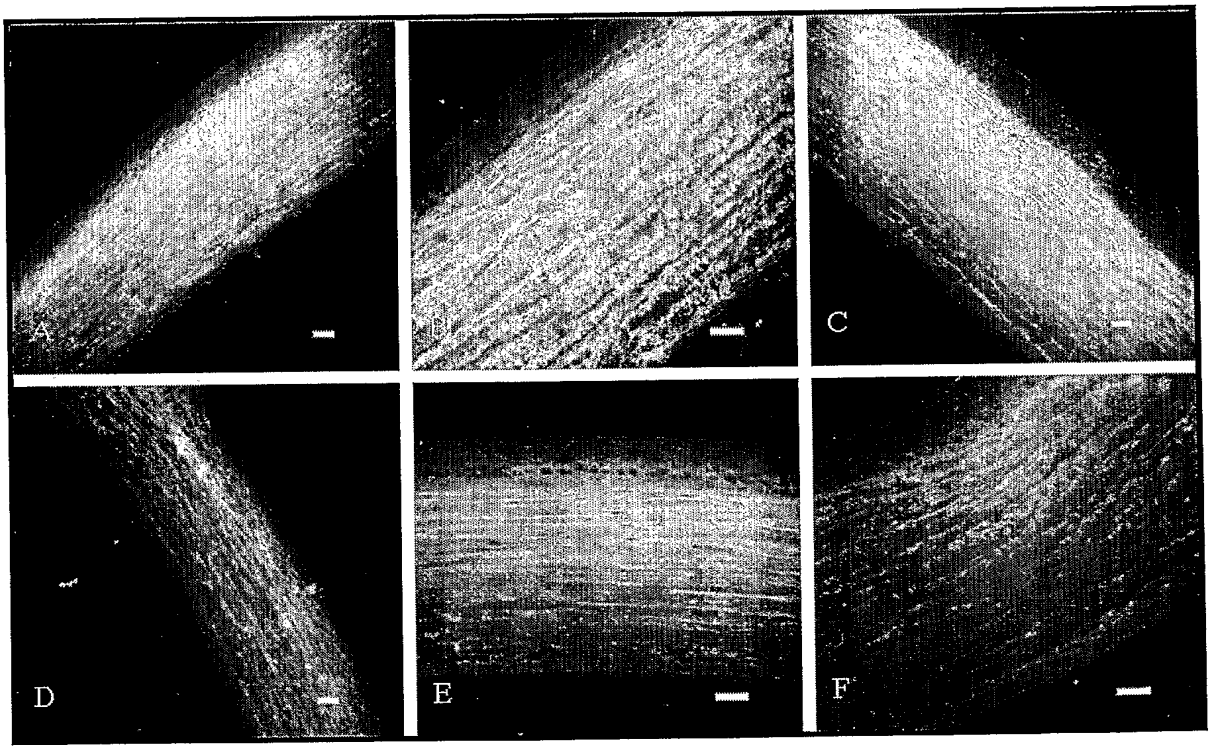

Figure 5

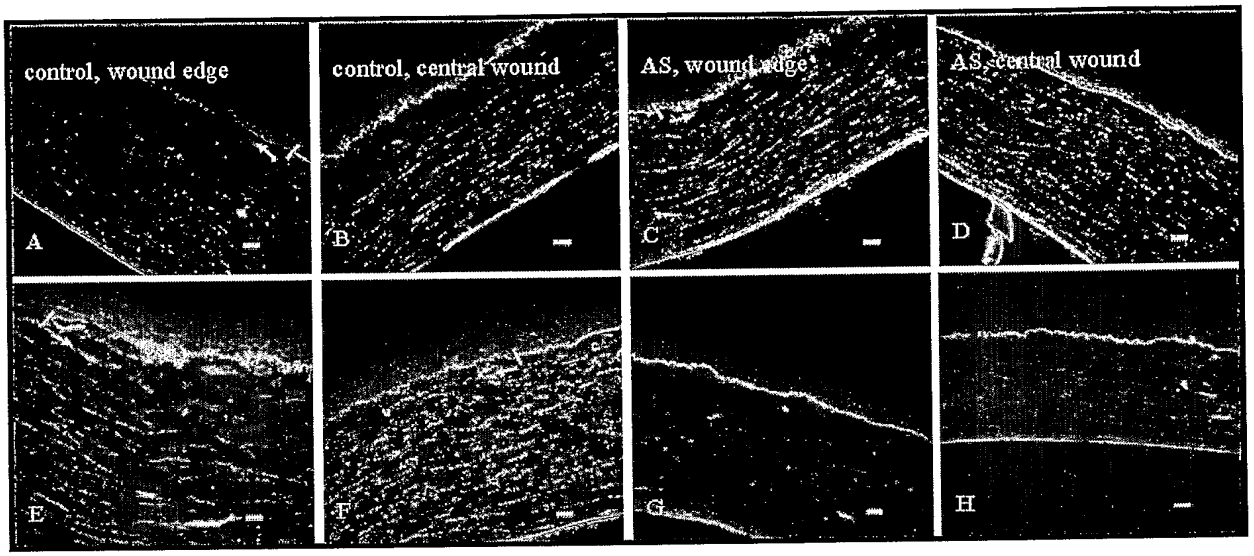

Figure 6

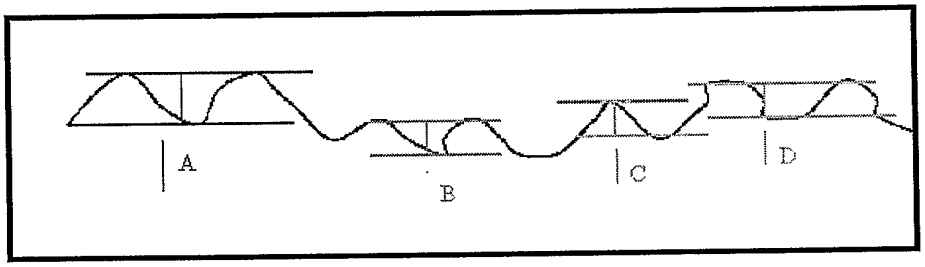

Figure 7